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19. Abstract

Polynitrophenols were used as model compounds for the metabolism of 2,4,6-trinitrotoluene. Oxidative as well as reductive initial reactions were observed during catabolism of polynitrophenols. The elimination of nitrite by an oxygenolytic mechanism was demonstrated with 2,6-dinitrophenol whereas 2,4-dinitrophenol or picric acid were subject to a nucleophilic reductive attack. The formation of a hydride-Meisenheimer complex followed by an elimination of nitrite leading to 2,4-dinitrophenol was also observed in cell-free systems.

2,4,6-trinitrotoluene was subject to a nucleophilic attack by a hydride ion leading to a Meisenheimer complex as the initial metabolite. The hydride-Meisenheimer complex of 2,4,6-trinitrotoluene was synthesized chemically as a reference and identified by spectroscopic methods.

In an anaerobic sludge supplemented with glucose and ammonia all nitro groups of 2,4,6-trinitrotoluene were reduced completely leading to 2,4,6-triaminotoluene which seemed to be further transformed under anaerobic conditions.

CONTENT

1. Introduction

The large scale manufacturing and handling of polynitroaromatic compounds as explosives resulted in an extensive contamination of soil and groundwater (1). Although these chemicals were synthesized and dumped mainly during World War II, they are still present at high concentrations.

Up to now little is known about the metabolism of polynitroaromatic compounds especially of 2,4,6-trinitrotoluene. In most cases cometabolic reactions are described by bacterial and fungal species leading to a variety of amino derivatives (2-11). Therefore, the aim of the project was to analyze the microbial degradative pathways of different nitroarenes as model compounds for 2,4,6-trinitrotoluene and to investigate the metabolism of 2,4,6-trinitrotoluene by microorganisms.
In the previous part of this project oxidative as well as reductive initial reactions were observed during catabolism of different nitroaromatic compounds. These mechanisms were studied in greater detail during the second part of the project:

1. The elimination of nitrite by an oxygenolytic mechanism was investigated with 2,6-dinitrophenol.

2. 2,4-dinitrophenol and picric acid (2,4,6-trinitrophenol were used as model compounds to elucidate a recently observed reductive hydride addition mechanism (12, 13) which appeared to be responsible for the liberation of nitrite.

Additionally, the catabolism of 2,4,6-trinitrotoluene was investigated under aerobic as well as under anaerobic conditions:

3. A main question of the present investigations was to clarify whether the reductive mechanism, which was observed during aerobic catabolism of picric acid, can also be adopted for the breakdown of 2,4,6-trinitrotoluene.

4. A two step anaerobic/aerobic treatment process was investigated for the elimination of nitroaromatics. After reduction of the (poly)nitroaromatic compound under anaerobic conditions the corresponding poly(amino)aromatic compound will be subject to hydrolytic and in the final aerobic process to oxidativ reactions.

2. 2,6-Dinitrophenol as a model compound for the oxidative elimination of nitrite

As described in the previous report of the current project an oxidative mechanism for the elimination of nitro groups as nitrite was observed in the 2,4-D degrading strain Alcaligenes eutrophus JMP 134 (14). This organism and its plasmid-free derivative JMP 222 were found to utilize 2,6-dinitrophenol as sole source of nitrogen (15) and under certain conditions 2,6-dinitrophenol was also used as a carbon and energy source. For the analysis of the degradative pathway of 2,6-dinitrophenol deficient mutants obtained by transposon mutagenesis were studied in greater detail. Two of these mutants still use 2,6-dinitrophenol as a nitrogen source, but no longer as a carbon source. One of mutants converted 2,6-dinitrophenol to 2-hydroxy-5-nitro-2,4-dienoic acid as a dead end metabolite and the other one to 4-nitropyroallol and nitrite. Whereas 4-nitropyrogallol was identified as an intermediate of 2,6-dinitrophenol by resting cell experiments of the parent strain Alcaligenes eutrophus JMP 222 resting cell experiments with 2-hydroxy-5-nitropenta-2,4-dienoic acid demonstrated that this compound seemed to a be dead end metabolite.

In accordance to these observations, the initial catabolic sequence shown in Fig. 1 was postulated. Obviously, two different mechanisms must exist for the elimination of nitrite from 2,6-dinitrophenol. Whereas the first nitro group is eliminated by an oxygenase leading 4-nitropyrogallol the liberation of the second nitro group must take place after ring cleavage.

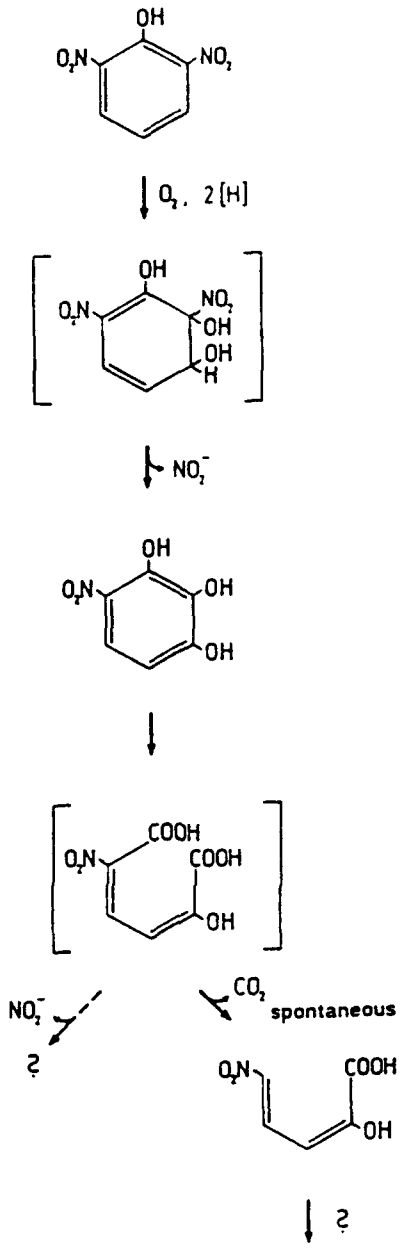

Figure 1: Proposed mechanism for the catabolism of 2,6-dinitrophenol by Alcaligenes eutrophus JMP 134 and 222

In order to elucidate the elimination of the second nitro group the following investigations were carried out:

1. Experiments in cell-free extracts

Although 2,6-dinitrophenol was not oxidized by cell-free extracts of Alcaligenes eutrophus JMP 222, ring cleavage of 4-nitro-

pyrogallol was observed by cell-free extracts of JMP 222: the disappearence of 4-nitropyrogallol correlated with the formation of a metabolite which seemed to be due to 2-hydroxy-5-nitromuconic acid (Fig. 1). The metabolite was further transformed to 2-hydroxy-5-nitropenta-2,4-dienoic acid in the presence but also in the absence of crude extract. The chemical instability of 2-hydroxy-5-nitromuconic acid explains the formation of 2-hydroxy-5-nitropenta-2,4-dienoic acid by spontaneous decarboxylation. The elimination of nitrite could not be demonstrated in crude extracts. Due to this instability the mechanism of the elimination of the nitro group could not yet be illucidated.

2. Transposon mutagenesis

To generate additional mutants which are blocked in the lower pathway of 2,6-dinitrophenol another transposon mutagenesis was carried out. Ecker et al. (15) isolated the following 36 mutants by Tn5 transposon mutagenesis (16):

A: auxotrophs (26)
B: mutants defective in the assimilation of nitrite (7)
C: mutants which were deficient in either a 2,6-dinitrophenol uptake system or in the initial step of 2,6-dinitrophenol degradation (2)
D: mutants which were blocked further downstream in the catabolic sequence (1)

The mutant type D (JMP222-α1d) which could use 2,6-dinitrophenol as a sole source of nitrogen but no longer as a carbon and energy source excreted 2-hydroxy-5-nitropenta-2,4-dienoic acid (Fig. 1). By use of Tn5 transposon mutagenesis mutants were enriched by penicillin or cycloserin treatment (17). From 9900 kanamycin-resistant mutants the following mutants were identified:

Type A: 18
Type B: 18
Type C: 1
Type D: 1

The type D mutant again excreted 2-hydroxy-5-nitropenta-2,4-dienoic acid as the mutant JMP222-α1d and the mutant of type C was not able to utilize 2,6-dinitrophenol as a nitrogen or carbon and energy source.

3. Initial reactions of picric acid metabolism

In the previous part of the project the catabolism of picric acid by two Rhodococcus erythropolis strains, HL 24-1 and HL 24-2 was studied. Both strains were isolated by their ability to utilize 2,4-dinitrophenol as a sole source of nitrogen. Both strains metabolize 2,4-dinitrophenol under concomitant liberation of stoichiometric amounts of nitrite and 4,6-dinitrohexanoate as minor metabolite giving evidence for a new and unexpected metabolic pathway (12)
One of these Rhodococcus erythropolis strains was also able to use picric acid as sole source of nitrogen after spontaneous mutation. Investigations on the biodegradation of picric acid demonstrated a reductive catabolic sequence for the elimination of nitrite (13). During growth of the mutant strain Rhodococcus erythropolis HL PM-1 an orange-red metabolite was observed.

This orange-red metabolite was unequivocally identified as a hydride-Meisenheimer complex of picric acid by comparison with a chemically generated hydride-Meisenheimer complex of picric acid in the previous part of the project. The identification of this complex shows that the aerobic catabolism of picric acid must be initiated by a nucleophilic attack of the aromatic ring by a hydride ion. The hydride-Meisenheimer complex was further metabolized by concomitant liberation of nitrite. The formation of the Meisenheimer complex by whole cells of Rhodococcus erythropolis HL PM-1 could also be demonstrated in cell-free extracts of this strain. Spectral changes between 280 and 600 nm observed during conversion of picric acid showed an increase of absorption at 500 nm (Fig. 2) indicating the formation of the hydride-Meisenheimer complex of picric acid. Simultaneously, the absorption maximum of picric acid (340 nm) declined. Picric acid

was transformed with a specific activity of 10 µmol/min x g protei

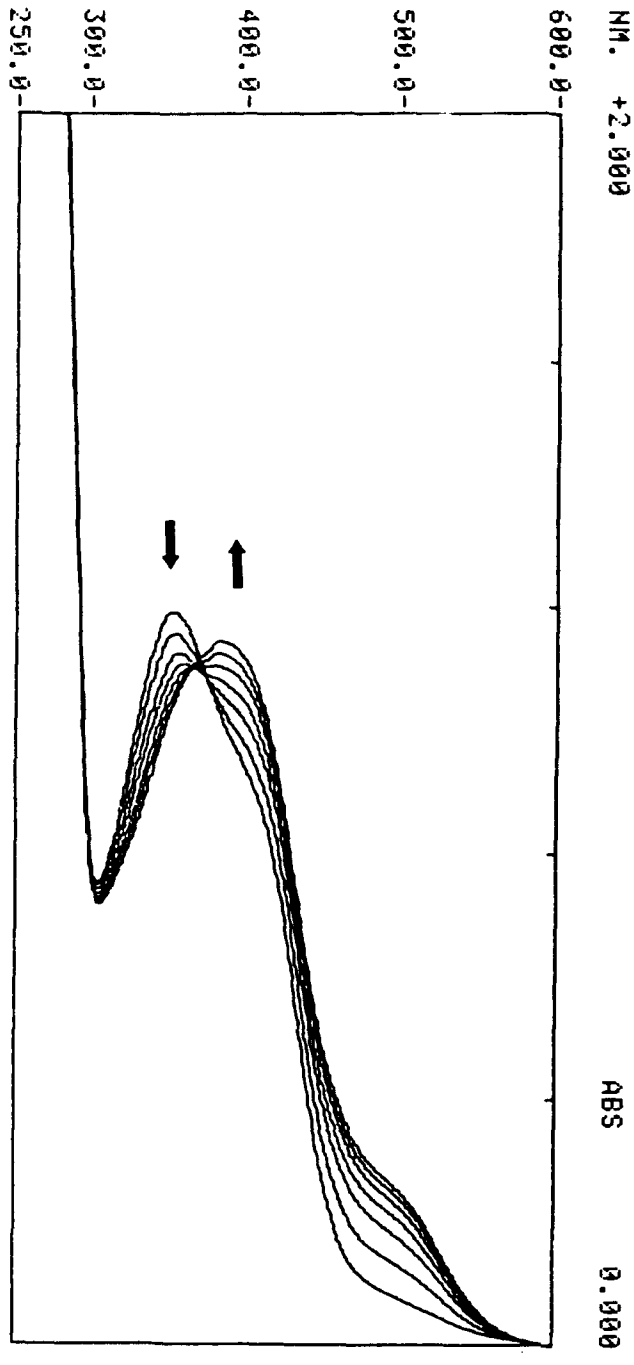

Figure 2: Spectral changes during turnover of picric acid by a cell-free extract of Rhodococcus erythropolis HL PM-1

Cell-free extract was added to a solution (final volume 1 ml) containing phosphate buffer (25 mM, pH 7.4) and 0.04 µmol picric acid.

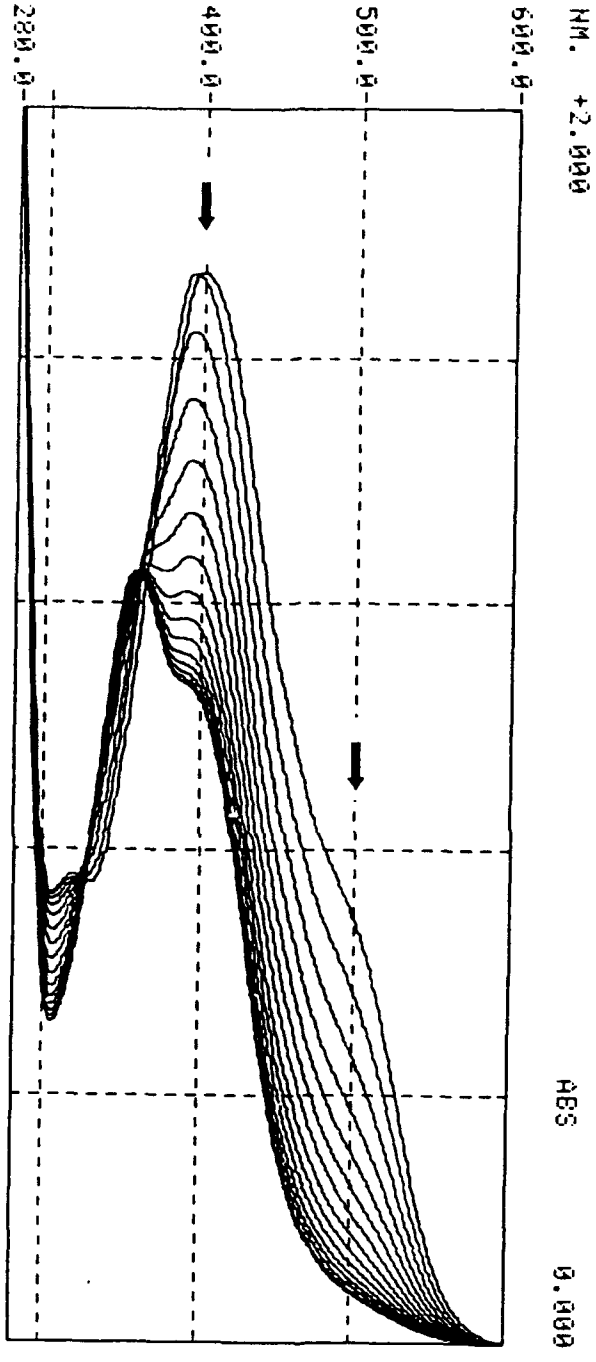

Figure 3: Conversion of the hydride-Meisenheimer complex of picric acid by a cell-free extract of Rhodococcus erythropolis HL PM-1

Cell-free extract was added to a solution (phosphate buffer, 25 mM, pH 7.4, final volume 1 ml) containing the hydride-Meisenheimer complex of picric acid which was obtained chemically by tetramethylammonium borohydride.

To elucidate the conversion of the hydride-Meisenheimer complex in cell-free extracts the complex was synthesized by use of tetramethylammonium borohydride. Spectral changes of the chemically obtained Meisenheimer complex by a cell-free extract of Rhodococcus erythropolis HL PM-1 were measured between 280 and 600 nm (Fig. 3). Complete conversion of the complex was characterized by an absorption decrease at 500 nm. Additionally, the absorption maximum shifted from 400 to 350 nm with a shoulder at 390 nm. This UV/Vis spectrum is due to authentic 2,4-dinitrophenol. It clearly demonstrates the reductive elimination of one nitro group from picric acid leading to 2,4-dinitrophenol.

The further bioconversion of 2,4-dinitrophenol was also investigated in-vitro. Thus spectral changes between 250 and 600 nm were followed in cell-free extracts of Rhodococcus erythropolis HL PM-1.

2,4-Dinitrophenol was converted with a specific acitivity of 6 μ mol/min gprotein. HPLC-analysis of the reaction mixture clearly showed the disappearance of 2,4-dinitrophenol and the concomitant formation of 4,6-dinitrohexanoate. 4,6-Dinitrohexanoate had already be identified as a minor metabolite during turnover of 2,4-dinitrophenol by growing or resting cells of Rhodococcus erythropolis HL 24-1 or HL 24-2 (wild-type of strain HL PM-1, 12).

Whether the disappearance of 4,6-dinitrohexanoate which was observed in cell-free extracts as well as by resting cells is due to biological or chemical transformation is subject of current investigations.

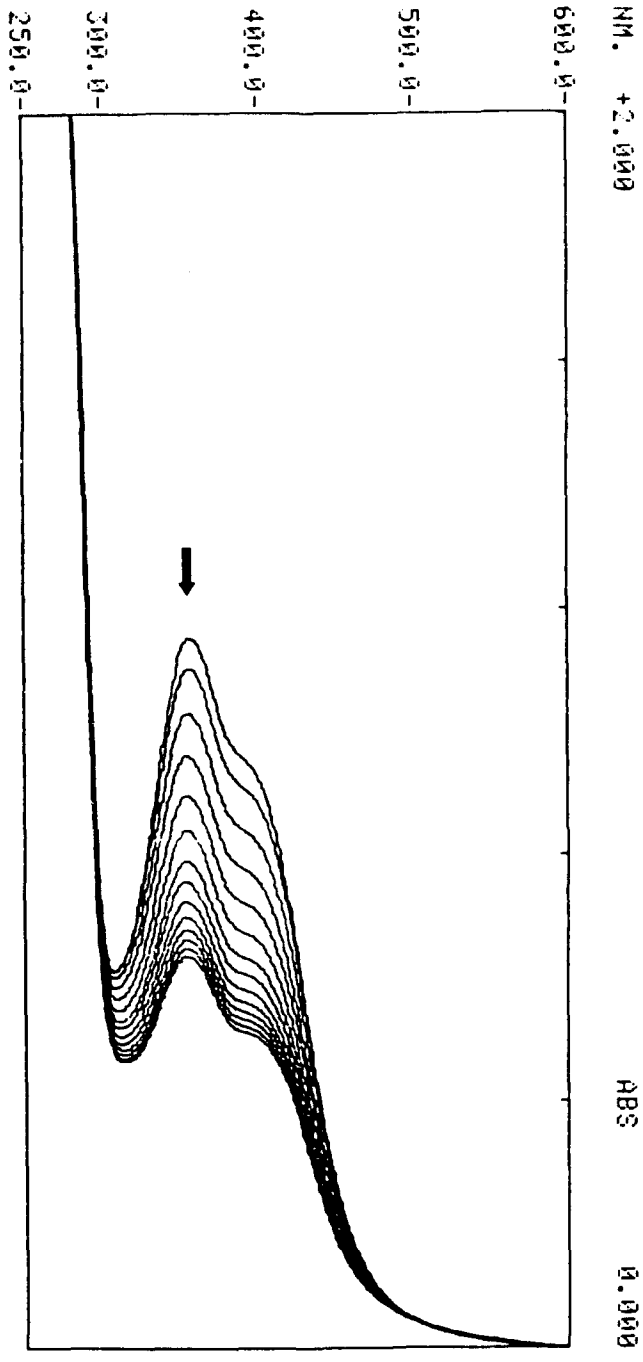

Figure 4: Spectral changes during the turnover of 2,4-dinitrophenol by a crude extract of Rhodococcus erythropolis HL PM-1

Cell-free extract was added to a solution (final volume 1 ml) containing phosphate buffer (25 mM, pH 7.4) and 0.04 µmol 2,4-dinitrophenol.

Present observations clearly demonstrate that the catabolism of picric acid is initiated by the addition of one hydride ion leading to a hydride-Meisenheimer complex followed by the elimination of nitrite and formation of 2,4-dinitrophenol which was further converted to 4,6-dinitrohexanoate (Fig. 5). The nucleophilic attack of the aromatic nucleus and finally its complete reduction by two additonal hydride equivalents can be explained by the highly electrophilic character of the polynitrosubstituted aromatic ring. This obviously prevents the well known electrophilic attack during aerobic metabolism of aromatic compounds.

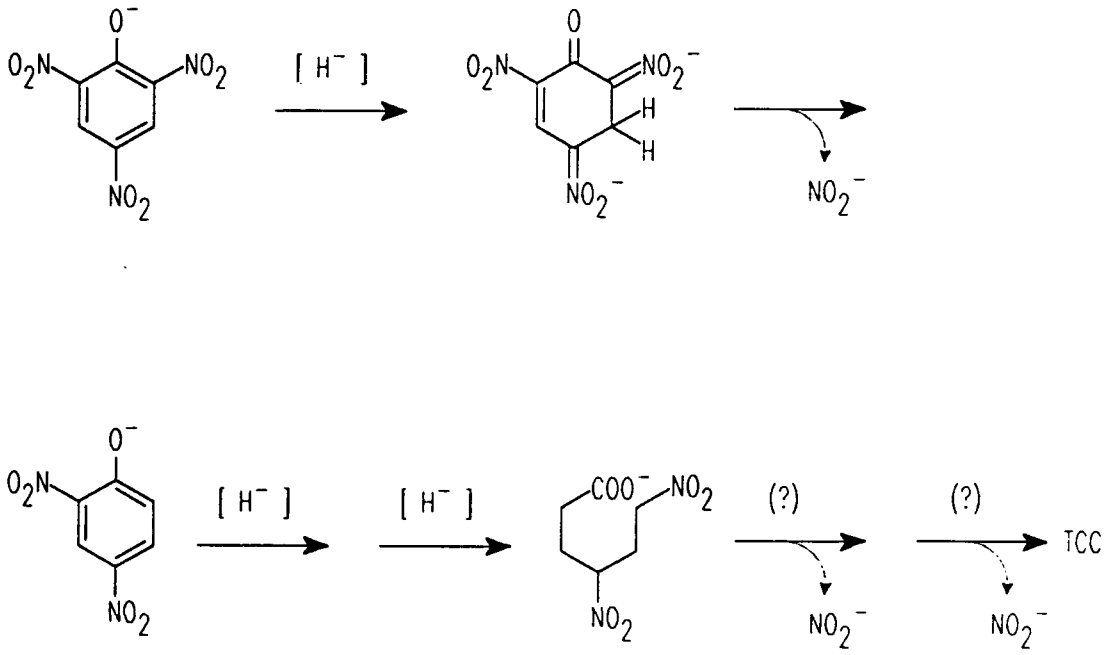

Figure 5: Transformations of picric acid and 2,4-dinitrophenol in cell-free systems of Rhodococcus erythropolis HL PM-1

4. Investigations on the aerobic metabolism of 2,4,6-trinitrotoluene

As described in the previous part of the project the formation of a hydride-Meisenheimer complex of 2,4,6-trinitrotoluene seems to play a role also in the metabolism of 2,4,6-trinitrotoluene. To confirm this observation the hydride-Meisenheimer complex of 2,4,6-trinitrotoluene was synthesized chemically as a reference compound. According to Kaplan and Siedle (18) the hydride-Meisenheimer complex of 2,4,6-trinitrotoluene was prepared at -10 °C from tetramethylammonium octahydrotriborate as a hydride donor. In contrast to the method of Kaplan and Siedle dark lustrous needles separated only after several hours. Thereof, the needles were collected by filtration after 24 h, washed with cold acetonitrile and dried in vacuo. Dissolved in acetonitrile, the isolated compound showed nearly the same absorption maxima as described by Kaplan and Siedle (18 , data in brackets): 255 nm (262 nm), 477 nm (478 nm) and 578 nm (582 nm). For final identification of the hydride-Meisenheimer complex of 2,4,6-trinitrotoluene ^{1}H and ^{13}C-NMR spectra were recorded. The ^{1}H-NMR data clearly demonstrated that exclusively the C3-hydride-Meisenheimer adduct (Fig. 6) was formed from 2,4,6-trinitrotoluene (Tab. 1, $-CH_2$ group at 3.90 ppm).

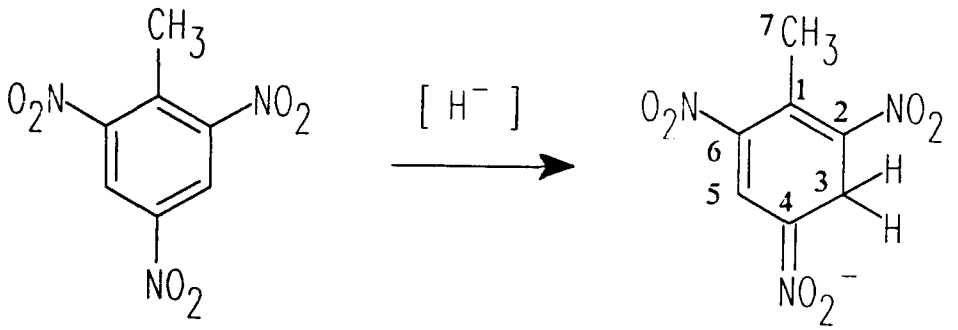

Figure 6: Formation of the C-3-hydride-Meisenheimer adduct of 2,4,6-trinitrotoluene. (Numbers indicate position of protons identified by NMR analysis, s. Tab. 1)

Additionally, the ^{1}H NMR data were in accordance with the NMR data obtained by Kaplan and Siedle (Tab. 1). The ^{13}C decoupled magnetic resonance data of the Meisenheimer complex were also compatible with the postulated C-3 adduct. One signal corresponds to the CH_2 group at 30.21 ppm and one signal to the methyl group at 18.72 ppm. The other five signals were due to five aromatic carbon atoms (signals at 141.26, 133.75, 129.67, 129.47, and 123.79 ppm). The carbons atoms of tetramethylammonium ion exhibited as a signal at 54.27 ppm.

Table 1: ^{1}H NMR data of the hydride-Meisenheimer complex of 2,4,6-trinitrotoluene

Nucleus	Chemical shift (DMSO)	Chemical shift# (DMSO)
3-H_a, H_a *	3.90 (dublet)	3.90
5-H_b *	8.37	8.38
7-$H_{(3)}$ *	2.57	no data
$(CH_3)_4$-N^+	3.10	3.12

data from Kaplan and Siedle (18)
* See Fig. 6
Tetramethylsilan was used a internal standard

For the identification of the hydride-Meisenheimer complex as a real metabolite of 2,4,6-trinitrotoluene it was necessary to analyse the stability of the complex under physiological conditions. The spontaneous chemical decomposition was accompanied by the generation of nonstoichiometric amounts of TNT (Fig. ', about 30 % of TNT were liberated). The half-life of the hydride-Meisenheimer complex under physiological conditions was about 5 h. Other products of the chemical decomposition in significant amounts could not be identified.

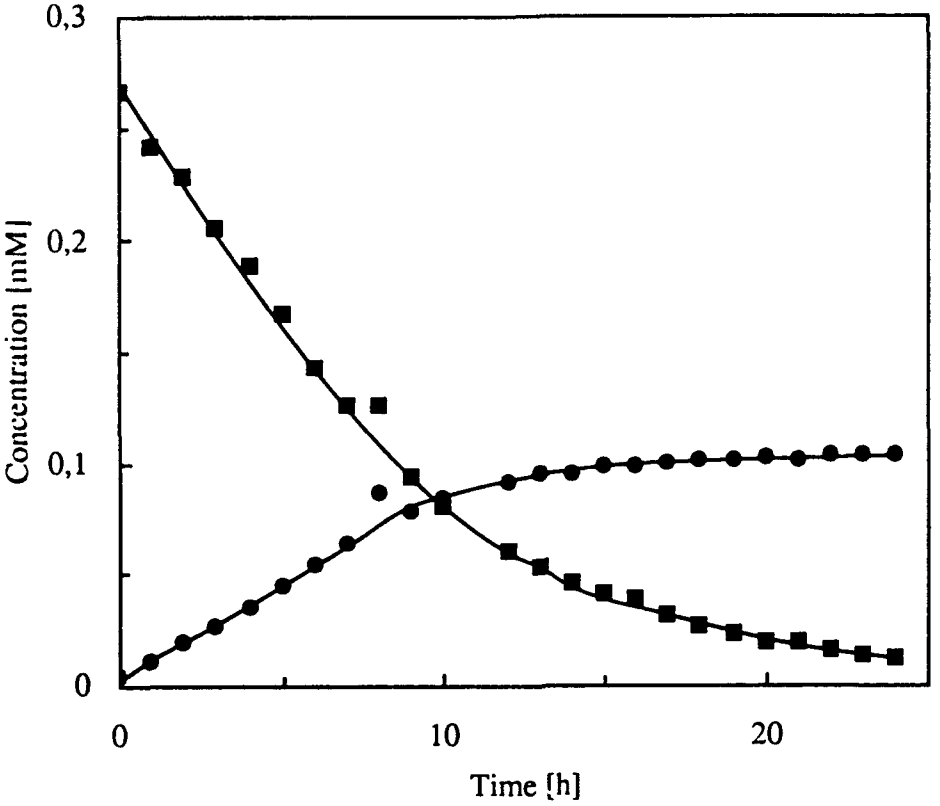

Figure 7: Spontaneous chemical decomposition of the hydride-Meisenheimer adduct under physiological conditions

The hydride-Meisenheimer complex was incubated in phosphate buffer at 30 °C. Concentration of the complex (■) and the formation of 2,4,6-trinitrotoluene (●) were followed by HPLC.

In order to identify the hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene resting cell experiments were carried out with the 4-nitrotoluene degrading Mycobacterium sp. HL 4-NT-1. During the conversion of 2,4,6-trinitrotoluene a dark red metabolite transiently accumulated. HPLC analysis (ion pair chromatography) showed that this metabolite had the same retention time (15,72 ml) and UV/visible absorption spectrum (λmax, 255, 477, and 578 nm) as the chemically obtained hydride-Meisenheimer complex (retention time, 15,97 min; absorption maxima at 255, 477, and 578 nm). Only 5 % of 2,4,6-trinitrotoluene in the reaction mixture were reduced to 4-amino-2,6-dinitrotoluene whereas about 40 % were liberated as the hydride-Meisenheimer complex inbetween indicating that the hydride addition mechanism plays an important role in the metabolism of 2,4,6-trinitrotoluene. Two additional metabolites transiently accumulated in the culture fluid (Fig. 10, retention

time at 3.3 and 6.9 min). The identification of both metabolites is subject of current investigations. After 75 min of the resting cell experiment (Fig. 8) 0.15 mM nitrite was realeased per 0.48 mM 2,4,6-trinitrotoluene. Although little amounts of nitrite were accumulated by resting cells of Mycobacterium sp. HL 4-NT-1 the organism could not utilize 2,4,6-trinitrotoluene as a nitrogen source.

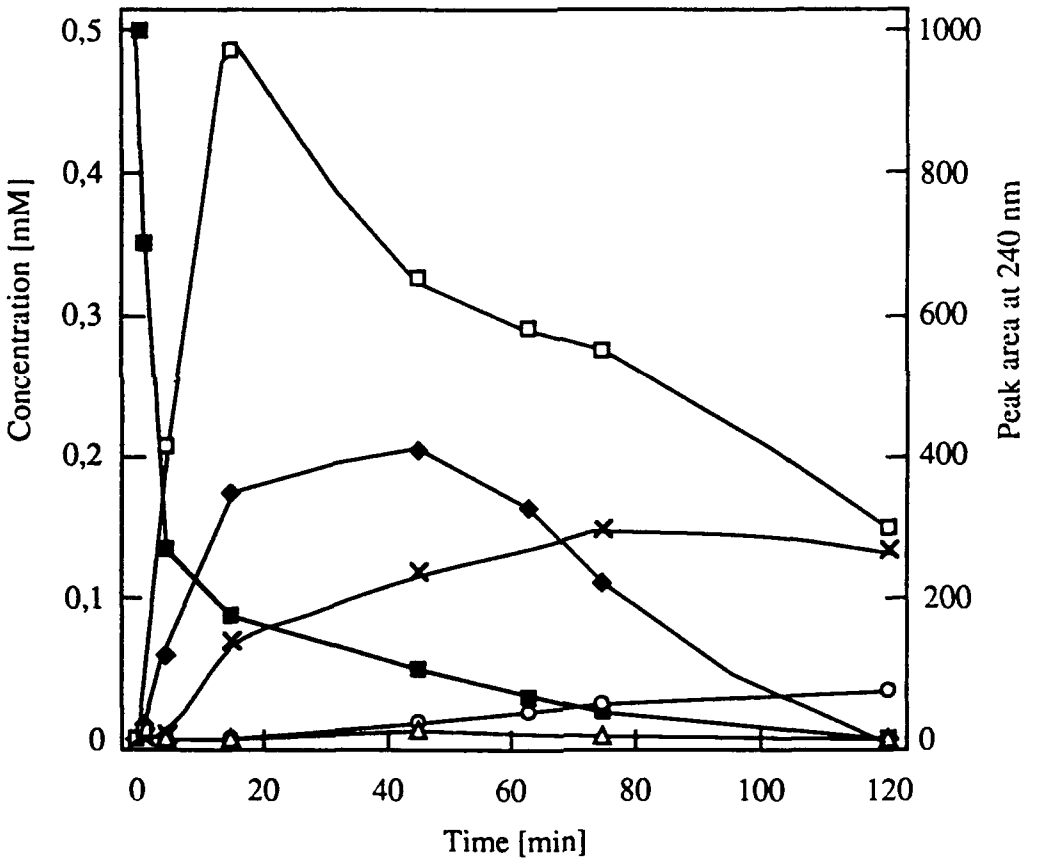

Figure 8: Conversion of 2,4,6-trinitrotoluene by resting cells of Mycobacterium sp. HL 4NT-1

Resting cells were obtained by growth in mineral medium with 4-nitrotoluene (0.5 mM) and succinate (10 mM) The cells were harvested, washed, and resuspended in phosphate buffer (optical density at 546 nm of 10), and incubated at 30°C with 0.5 mM 2,4,6-trinitrotoluene on a water bath shaker. Concentration of 2,4,6-trinitrotoluene (■), the Meisenheimer complex (◆), 4-amino-2,6-dinitrotoluene (○) and not yet identified metabolites Rt 6.7 (□) and Rt 3.3 (△) were determined by HPLC (ion pair chromatography), whereas nitrite (x) was determined by reverse phase chromatography.

As described in the previous report the isolation of 2,4,6-trinitrotoluene degrading microorganisms was successfull by enrichment cultures using saturated solutions of 2,4,6-trinitrotoluene (about 0.6 mM) as a nitrogen source. This indicates that 2,4,6-trinitrotoluene is considerably less toxic for these microorganisms than expected from literature (4). One of the isolated strains CV TNT-8 utilizing 2,4,6-trinitrotoluene as a nitrogen source excreted small amounts of the hydride-Meisenheimer complex during growth experiments. For the other strains, however, the Meisenheimer complex could not yet be detected as a metabolite in the catabolism of 2,4,6-trinitrotoluene. Current research foccusses on the further bioconversion of the hydride-Meisenheimer complex and on the identification of metabolites excreted during conversion of 2,4,6-trinitrotoluene by resting cells of Mycobacterium sp. HL 4-NT-1 and the 2,4,6-trinitrotoluene degrading microorganisms.

5. An anaerobic/aerobic process for the microbial breakdown of 2,4,6-trinitrotoluene by microorganisms

As an alternative of 2,4,6-trinitrotoluene degradation by microorganisms anaerobic and aerobic treatment process developed. Firstly, nitrobenzene as a model compound for this strategy was shown to be biodegradable by an anaerob/aerob process. Under anaerobic conditions anaerobic sludge gratuitously reduced nitrobenzene to aniline in equimolar amounts, which was mineralized by activated sludge under aerobic conditions (20). As described recently in literature (21) 2,4,6-trinitrotoluene could be reduced to 2,4,6-triaminotoluene by a sulfate reducing microorganism. In the previous part of the project this reduction was also demonstrated by an anaerobic sludge consortia without addition of reducing agents. The reduction of 2,4,6-trinitrotoluene to 2,4,6-triaminotoluene occurred via 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene as shown in Fig. 9.

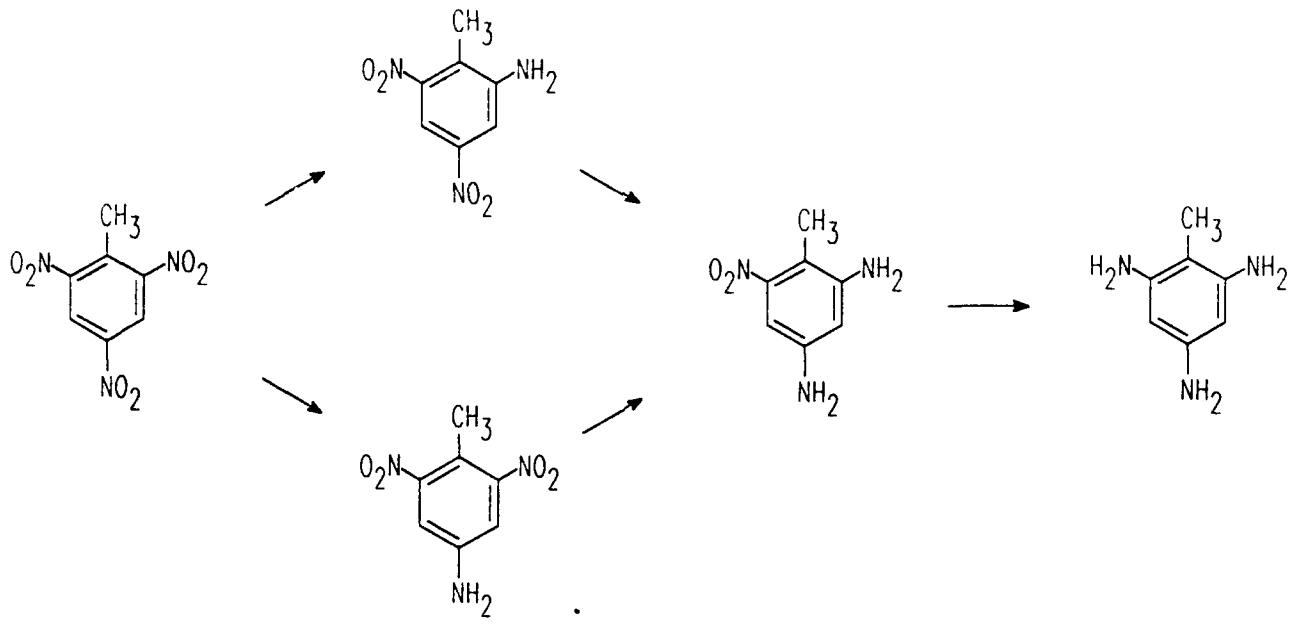

Figure 9: Reduction sequence of 2,4,6-trinitrotoluene by microorganisms under anaerobic conditions

Fig. 10 shows the reduction of 2,4,6-trinitrotoluene by anaerob sludge. The reaction mixture was supplemented with glucose as carbon and energy source and ammonia as nitrogen source. The experiment was carried out at a pH of about 7.4. This avoids hydrolytic destruction of 2,4,6-triaminotolunene during acidification of the culture medium (22). Under these conditions 2,4,6-trinitrotoluene was reduced almost quantitativly to 2,4,6-triaminotoluene. A slow disapperance of 2,4,6-triaminotoluene was observed during this experiment indicating further degradation of 2,4,6-triaminotoluene under anaerobic conditions. Up to now enrich..ents for the productive degradation of 2,4,6-triaminotoluene under N-limiting and anaerobic conditions as well as under anoxic (denitrifying) conditions failed. Therefore current work focusses on the fate of 2,4,6-triaminotoluene in an anaerobic sludge supplemented with glucose and ammonia.

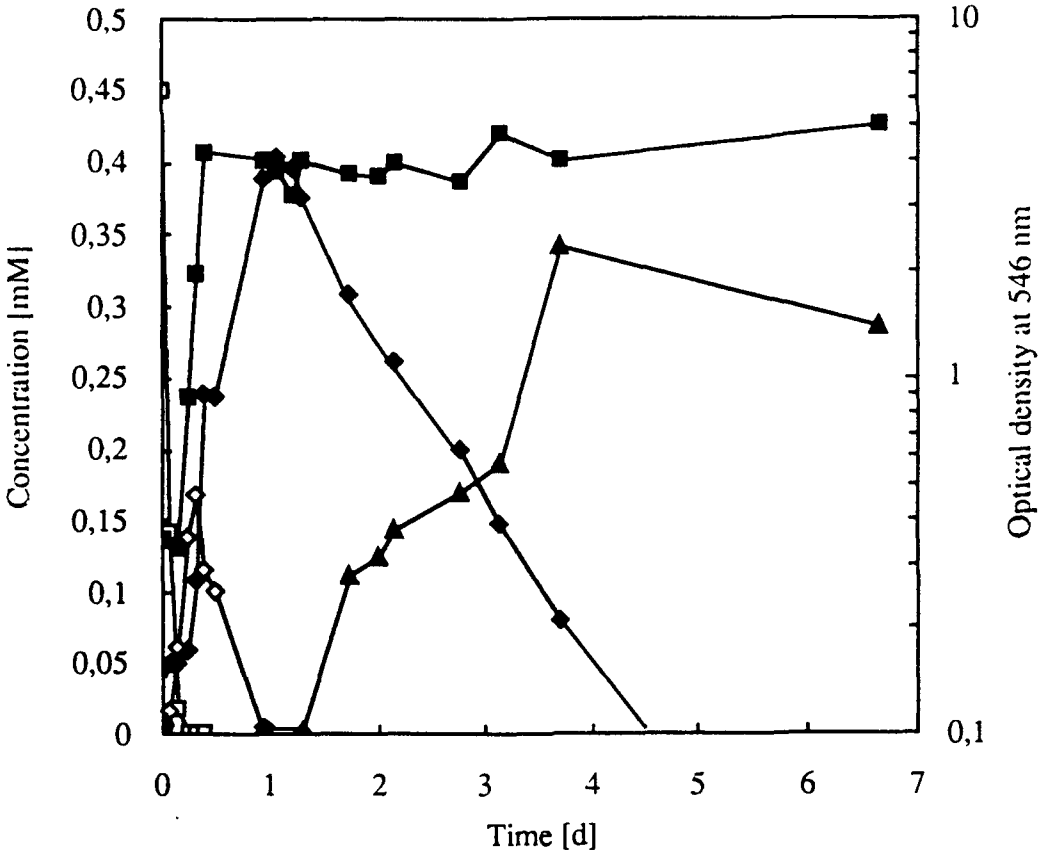

Figure 10: Turnover of 2,4,6-trinitrotoluene by an anaerobic sludge

In an argon atmosphere anaerobic sludge was incubated in mineral salts medium supplemented with 0.5 mM 2,4,6-trinitrotoluene and 40 mM glucose on a rotary shaker at 30°C.
The concentration of 2,4,6-trinitrotoluene (□), 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene (◇), 2,4-diamino-6-nitrotoluene (◆), and 2,4,6-triaminotoluene (▲) was determined by HPLC analysis. The optical density (■) was measured at 546 nm.

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Interactions

Oral presentation at the 100. WE-Heraeus-Seminar in Bad Honnef, Germany:
H. Lenke, P.-G. Rieger, C. Vorbeck, and H.-J. Knackmuss (1992) Initial hydrogenation during aerobic catabolism of polynitro-aromatic compounds

Two poster presentations at the VAAM Meeting in Leipzig, Germany:

P.-G. Rieger, H. Lenke, and H.-J. Knackmuss (1993) Aerobic bacterial degradation of 2,4,6-trinitrophenol (picric acid): reductive initial attack.

C. Vorbeck, H. Lenke, and H.-J. Knackmuss (1993) Aerobic metabolism of 2,4,6-trinitrotoluene (TNT).